UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

DATE: May 8, 2014

SUBJECT: **FLUOPYRAM:** Report of the Cancer Assessment Review Committee

PC Code: 080302	**DP Barcode:** N/A
Decision No.: N/A	**Registration No.:** N/A
Petition No.: N/A	**Regulatory Action:** N/A
Risk Assessment Type: NA	**Case No.:** N/A
TXR No.: 0056961	**CAS No.:** N/A
MRID No.: N/A	**40 CFR:** N/A

FROM: Ronnie J. Bever Jr., PhD, DABT, Toxicologist
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THROUGH Jess Rowland, Chair
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TO: Whang Phang, Ph.D., Senior Toxicologist
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The Cancer Assessment Review Committee (CARC) met on February 26, 2014 to evaluate the cancer classification of fluopyram in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the final Cancer Assessment Document.

CANCER ASSESSMENT DOCUMENT

EVALUATION OF THE CARCINOGENIC POTENTIAL OF

Fluopyram

PC CODE: **080302**

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE

HEALTH EFFECTS DIVISION

OFFICE OF PESTICIDE PROGRAMS

May 8, 2014

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EXECUTIVE SUMMARY

On February 26, 2014, the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP) evaluated the carcinogenic mode of action data provided by the Registrant for fluopyram. This is the second CARC meeting for fluopyram. Fluopyram is a pesticide active ingredient that is used to control fungal diseases of grapes and tomatoes. Fluopyram has been proposed for use on ornamentals and non-residential turf.

In 2009, the CARC classified fluopyram as "Likely to be Carcinogenic to Humans" based on tumors in two species and two sexes (treatment-related increases in thyroid follicular cell adenomas in male mice and liver tumors in female rats). At that time, the Registrant also submitted a proposed mode of action (MOA) for the liver and thyroid tumors. The CARC determined the submitted MOA data were insufficient to establish a mode of action for the observed tumors, in accordance with the framework provided by the International Programme on Chemical Safety (IPCS). The major deficiencies were the lack of dose-response concordance with key events and tumors; and the studies were performed at doses in excess of the dose that caused tumors in the carcinogenicity studies. The CARC recommended the use of a linear low dose extrapolation model applied Q_1* for quantitative estimation of human risk.

Since the 2009 CARC meeting, the Registrant has submitted a series of studies to support a postulated MOA for liver and thyroid tumor formation. The submitted data were considered adequate to establish the mode of action for the etiology of these tumors. The results of the new studies are discussed in this report.

Key events leading to the progression towards liver tumors included sequentially the activation of the CAR/PXR receptors resulting in induction of hepatic cytochrome P450 activity, hepatocellular proliferation, altered hepatic foci, and liver tumors. These key events were established based on dose-response and temporal concordance at appropriate doses.

Key events leading to the progression towards thyroid tumors included sequentially the activation of the CAR/PXR receptors resulting in induction of hepatic cytochrome P450 activity, induction of Phase II hepatic enzymes resulting in increased serum T4 clearance, increased TSH, increased thyroid cell proliferation, increased thyroid cell hyperplasia, and thyroid tumors. These key events were established based on dose-response and temporal concordance at appropriate doses.

In accordance with the IPCS framework, alternate modes of actions were also considered, but were rejected based on the available toxicology data and published literature.

The CARC classified fluopyram as "Not Likely to be Carcinogenic to Humans" at doses that do not induce cellular proliferation in the liver or thyroid glands. This classification was based on convincing evidence that non-genotoxic modes of action for liver tumors in rats and thyroid tumors in mice have been established and that the carcinogenic effects have been demonstrated as a result of a mode of action dependent on activation of the CAR/PXR receptors.

The CARC has determined that quantification of risk is not required. There is sufficient data to ascertain the mode of action of fluopyram. The chronic Reference Dose (RfD) is derived using the NOAEL of 1.2 mg/kg/day as the "point of departure" which is below the dose of 11

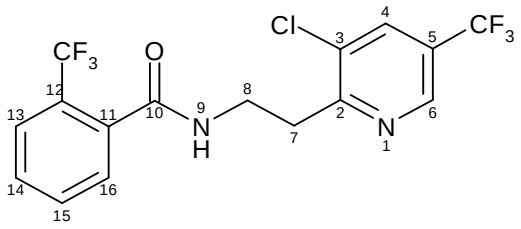
mg/kg/day that caused cell proliferation in the liver (i.e., a key event in tumor formation) and the subsequent liver tumors at a higher dose (89 mg/kg/day). Additionally, there is no concern for mutagenicity.

I. INTRODUCTION

On February 26, 2014, the CARC evaluated the mode of action studies submitted by the Registrant in context with the carcinogenic potential of fluopyram. Previously, on July 8, 2009, the CARC had evaluated the carcinogenic potential of fluopyram (TXR No.0055261).

II. BACKGROUND INFORMATION

Fluopyram is a new broad-spectrum systemic fungicide of the carboxamide group (FRAC Group 7). It acts on cell respiration in the fungus by inhibiting succinate dehydrogenase (mitochondrial respiration Complex II), thus blocking electron transport. The main use of fluopyram is the selective control of a variety of fungal diseases (like powdery mildew species, *Botrytis cinerea* and *Alternaria solani)* on grape vines and tomatoes. It is also proposed to be used on ornamentals and non-residential turf. Its structure and other pertinent information are depicted in Table 1.

Table 1: Structure and Chemical Information for Fluopyram	
Chemical structure:	
Empirical formula:	$C_{16}H_{11}ClF_6N_2O$
Common name:	Fluopyram
CAS name:	Benzamide, N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)-(9CI)
CAS no.:	658066-35-4
PC Code	080302

III. EVALUATION OF CARCINOGENICITY STUDIES

A summary of the conclusions of the July 8, 2009 CARC meeting is provided below.

In a combined chronic toxicity and carcinogenicity study, groups of 60 male and female Wistar rats were fed a diet containing 0, 30, 150, and 750 ppm (males) and 0, 30, 150, and 1500 ppm (females) fluopyram for 24 months. In males, the top dose level of 750 ppm had to be reduced to 375 ppm from Week 85 onwards, because of the high mortality in this group. Over the whole study period, these dietary concentrations corresponded to a mean daily intake of 0, 1.20, 6.0, and 29 mg/kg bw in male rats or 1.68, 8.6, and 89 mg/kg bw in females.

Administration of fluopyram resulted in the induction of liver tumors in female Wistar rats. There were statistically significant trends for liver adenomas ($p<0.01$), carcinomas ($p<0.05$), and combined liver adenomas and carcinomas ($p<0.01$). There were significant pair-wise comparisons of the 1500 ppm dose group with the controls for liver adenomas at $p<0.05$ and for combined liver adenomas and carcinomas at $p<0.01$. When compared to historical control data (uncensored data) from the testing laboratory, the incidence of hepatocellular adenomas in the

female high dose group (9/55, 16%) was outside the range of the historical control group (range, 0 - 5%; average, 1.9%). Similarly, the incidence of hepatocellular carcinomas in the female mid (2/56, 4%) and high dose groups (3/55, 5%), while not statistically significant by pair-wise comparison, exceeded the range of the historical control group (no carcinomas observed in 10 studies from 2000 - 2006) and was considered to be biologically relevant. There were no statistically significant trends or significant pair-wise comparisons of the dosed groups with the controls for the male rats. **The CARC considered the liver tumors in female Wistar rats to be treatment-related.**

Groups of 60 male and female C57BL/6J mice were fed diets containing 0, 30, 150, or 750 ppm of fluopyram (corresponding to a mean compound intake of 0, 4.2, 20.9, and 105 mg/kg bw/day in males and 0, 5.3, 26.8, and 129 mg/kg bw/day in females, respectively) for up to 78 weeks.

Administration of fluopyram resulted in the induction of thyroid follicular cell tumors in male C57BL/6J mice. Male mice had a statistically significant trend at $p<0.01$ and a significant pair-wise comparison of the 750 ppm dose group with the controls at $p<0.05$ for thyroid follicular cell adenomas. There were no statistically significant trends or significant pair-wise comparisons of the dosed groups with the controls for the female mice. When compared to historical control data (uncensored data) from the testing laboratory, the incidence of thyroid follicular cell adenomas in the male high dose group (7/48, 15%) was outside the range of the historical control group (range, 0 - 2%; average, 0.4%). **The CARC considered the thyroid follicular cell adenomas in male C57BL/6J mice to be treatment-related.**

The CARC classified it as **"Likely to be Carcinogenic to Humans"** based on tumors in two species and two sexes: a treatment-related increase in thyroid follicular cell adenomas in high dose male mice and liver tumors in high dose female rats (Tables 2 and 3). There was no mutagenic concern for fluopyram, and CARC recommended the use of a linear low dose extrapolation model (Q_1*) for quantitative estimation of human cancer risk. The unit risk, Q_1^* $(mg/kg/day)^{-1}$ was determined to be 1.55×10^{-2} in human equivalents based upon female rat liver combined adenoma and carcinoma tumor rates (TXR No. 0055261).

At the meeting, the CARC reviewed the proposed MOA data submitted by the Registrant for liver tumors in female rats and thyroid follicular cell adenomas in male mice. The CARC determined that the submitted data were insufficient to establish a MOA for the observed tumors. The major deficiencies identified were the lack of dose-response concordance with key events and tumors, and the doses tested in the MOA studies were above the doses that caused tumors in the carcinogenicity studies.

Table 2. Liver Tumor Rates in Female Rats and Fisher's Exact Test and Exact Test for Trend Results. [a]				
Dose (ppm)	**0**	**30**	**150**	**1500**
Adenomas	2/59	2/57	0/56	9 [b]/55
(%)	(3)	(4)	(0)	(16)
p =	0.00049**	0.67765	1.00000	0.01978*
Carcinomas	0/59	0/57	2/56	3 [c]/55
(%)	(0)	(0)	(4)	(5)
p =	0.02134*	1.00000	0.23494	0.10910
Combined	2/59	2/57	2/56	11 [d]/55
(%)	(3)	(4)	(4)	(20)
p =	0.00015**	0.67765	0.67083	0.00536**
Historical controls	Hepatocellular adenomas: range, 0 – 5%; average, 1.9% Hepatocellular carcinomas: no carcinomas observed in 10 studies from 2000 to 2006.			

[a] Data were obtained from page 10 of the previous Fluoropyram CARC report (TXR No. 0055261; 11/25/2009). Data are reported as the number of tumor bearing animals/number of animals examined, excluding those that died or were sacrificed before Week 54.

[b] First adenoma observed at Week 75 at 1500 ppm.

[c] First carcinoma observed at Week 97 at 1500 ppm.

[d] One animal in the 1500 ppm dose group had both an adenoma and a carcinoma.

Significance of trend denoted at control. Significance of pair-wise comparison with control denoted at dose level.

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

Table 3. Thyroid Follicular Cell Tumor Rates and Fisher's Exact Test and Exact Test for Trend Results in Male Mice. [a]				
Dose (ppm)	**0**	**30**	**150**	**750**
Adenomas[#]	1/49	1/47	3 [b]/48	7/48
(%)	(2)	(2)	(6)	(15)
p =	0.00357**	0.74211	0.30076	0.02758*
Historical controls	Thyroid adenomas: range, 0-2%; average, 0.4%			

[a] Data were obtained from page 14 of the previous Fluoropyram CARC report (TXR No. 0055261; 11/25/2009). Data are reported as the number of tumor bearing animals/number of animals examined, excluding those that died or were sacrificed before Week 54.

[b] First adenoma observed at Week 79 at 150 ppm.

Significance of trend denoted at control. Significance of pair-wise comparison with control denoted at dose level.

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

Subsequent to the 2009 CARC meeting, the registrant conducted a series of studies to support a postulated MOA for liver and thyroid tumor formation. The citation and summary of these studies are presented in Attachment A. The results of these new studies are discussed in the context of the proposed MOA.

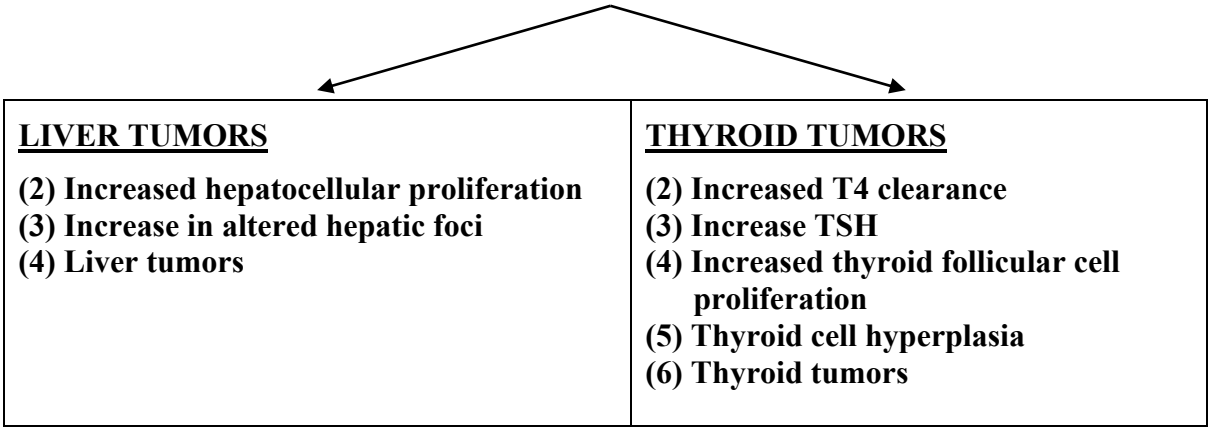
IV. MODE OF ACTION STUDIES

It should be noted that most of the information presented below is partially derived from the submission (MRID 49005912).

The results of the battery of genetic toxicity studies demonstrate that fluopyram is not mutagenic or genotoxic. For non-DNA-reactive rodent liver carcinogens, several MOAs have been identified that act by stimulating hepatocellular proliferation through either a receptor- or non-receptor-mediated mechanism. Evidence from early studies suggested that the profile of liver effects induced by fluopyram were similar to those induced by phenobarbital, a chemical known to exert its effects through activation of the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) in the liver. Compounds that induce both CAR/PXR can also disrupt thyroid hormone balance, leading to stimulation of the thyroid cells, which can eventually result in formation of thyroid tumors following chronic exposure (Hiasa *et al.*, 1982; McClain *et al.*, 1988). Based on this information, the registrant proposed a MOA for liver and thyroid tumor formation as illustrated in the following diagram.

Proposed Key Events for CAR/PXR-Mediated MOAs for Liver and Thyroid Tumors.

(1) CAR/PXR receptor activation in liver
Via altered hepatic gene expression and
induction of hepatic Phase I and II enzymes



Data were obtained from page 12 of the MOA Document (MRID 49005912). It should be noted that Key Events 1 and 2 for the liver; and Key Events 1 and 4 for the thyroid are mostly reversible on discontinuance of treatment (see MRIDs 49005902 and 49005911)

The submitted Mechanistic/MOA studies were conducted to demonstrate these key events occur following exposure to fluopyram as well as to characterize the dose- and temporal-response relationships for each of the key events. As liver tumors occurred only in the female rat and

thyroid tumors only in the male mouse, the mechanistic studies reported in the MOA document focused primarily on liver tumors in female rats and thyroid tumors in male mice to reduce the number of animals employed to generate the data to support the proposed MOA of fluopyram.

a. Liver Tumor MOA and Key Events

i. Key Event #1: CAR/PXR Receptor Activation (induction of hepatic cytochrome p450 gene expression and enzyme activity)

The first key event is activation of CAR/PXR nuclear receptors. However, no data are available for fluopyram that show direct interaction between fluopyram and CAR/PXR nuclear receptor. Evidence for CAR/PXR receptor activation can be provided by demonstrating increased expression of specific hepatic cytochrome P450 genes and activation of their associated enzyme products. In particular, activation of CAR/PXR is associated with the induction of the *Cyp2b* (CAR) and *Cyp3a* (PXR) families (Ueda *et al.*, 2002) and their corresponding enzymes, as measured by pentoxyresorufin-O-depentylation (PROD) and benzyloxyresorufin-O-debenzylation (BROD) or benzyloxyquinoline (BQ). For example, the prototypical CAR/PXR inducer is phenobarbital, which produces marked increases in these parameters in rodents following exposure.

For fluopyram, data showing CAR/PXR activation are provided from two new mechanistic studies where quantitative polymerase chain reaction (qPCR) and enzyme activity were used to characterize the dose and temporal response of Phase I (AhR-, CAR-, PXR-, or PPARα-associated) transcripts/enzymes in female rats following treatment with fluopyram. In the first study (MRID 49005910), the animals were exposed to fluopyram in the diet for either 3 or 7 days. In the second study (MRID 49005902), the animals were exposed for 28 days with additional control and high dose group females being maintained on control diet for an additional 28 days after the exposure period. The dietary levels used in these studies covered the range of levels tested in female rats in the chronic cancer bioassay, *i.e.,* 30, 150, and 1500 ppm. Summaries of the gene expression data and the enzyme activity data are presented in Tables 6 and 7, respectively.

Gene Expression

Table 4 presents the data showing Phase I gene expression which demonstrates a dose-response increase in CAR/PXR-related genes (*Cyp2b1* and *Cyp3a3*) due to fluopyram treatment. Additionally, elevated transcript levels of *Cyp1a1* were seen with fluopyram, suggesting possible direct activation of the AhR. *Cyp4a1* was not elevated in these studies, suggesting the lack of PPARα induction.

A return to normal transcript levels for Phase I enzyme-related genes was recorded for animals exposed to fluopyram for 28 days at the dose that caused tumors and then placed on a control diet for a further 28 days. Overall, the results of these gene expression studies demonstrated dose and temporal responses (3–28 days) of CAR/PXR at ≥ 150 ppm.

Table 4. Gene Expression Presented as Mean Fold Change Relative to Controls for Female Rats Exposed to Fluopyram for 3, 7, or 28 Days with a Recovery Group of 28 Days. [a]

	Dose (ppm)	0 n = 15	30 n = 15	75 n = 15	150 n = 15	600 n = 15	1500 [b] n = 15
Associated receptors	**Rat genes**	**3-Days dosing**					
AhR	***Cyp1a1***	1.35 ± 1.14	1.12 ± 0.68	1.51 ± 1.61	2.30 ± 2.30 (1.7)	9.87 ± 8.29** (7.3)	84.6 ± 51.6** (62.7)
CAR	***Cyp2b1***	1.27 ± 1.87	0.81 ± 0.56	1.44 ± 0.86	4.2 ± 3.38** (3.3)	63.0 ± 71.2** (49.6)	309.5 ± 176.7** (244.1)
PXR	***Cyp3a3***	0.93 ± 0.49	1.01 ± 0.50	1.38 ± 0.68* (1.9)	2.41 ± 0.89** (2.6)	7.64 ± 2.23** (8.2)	20.0 ± 6.45** (21.5)
PPARα	***Cyp4a1***	1.46 ± 0.33	1.37 ± 0.38	1.51 ± 0.40	1.30 ± 0.40	1.43 ± 0.46	1.14 ± 0.27
Associated receptors	**Rat genes**	**7-Days dosing**					
AhR	***Cyp1a1***	2.26 ± 1.30	3.07 ± 3.87	4.00 ± 3.87	10.31 ± 1.01** (4.6)	143.7 ± 81.0** (63.6)	503.8 ± 170.5** (222.9)
CAR	***Cyp2b1***	0.95 ± 0.74	2.43 ± 1.85	2.93 ± 3.87	13.64 ± 2.06** (14.4)	310.2 ± 22.0** (326.5)	1362.3 ± 1422.2** (1434)
PXR	***Cyp3a3***	0.99 ± 0.59	1.46 ± 0.84	1.93 ± 0.79** (1.9)	3.59 ± 1.65** (3.6)	12.32 ± 3.75** (12.4)	28.27 ± 10.15** (28.6)
PPARα	***Cyp4a1***	0.73 ± 0.26	0.70 ± 0.22	0.62 ± 0.14	0.64 ± 0.24	0.64 ± 0.19	0.46 ± 0.11**
Associated receptors	**Rat genes**	**28-Days dosing**					
AhR	***Cyp1a1***	1.06 ± 0.70	1.87 ± 1.25	2.43 ± 1.48	8.60 ± 7.02** (8.1)	107 ± 28.0** (100.9)	375 ± 130** (354.7)
CAR	***Cyp2b1***	1.26 ± 1.15	3.38 ± 7.17	2.09 ± 1.91	13.7 ± 10.6** (10.9)	268 ± 193** (212.5)	1345 ± 1518** (1543.8)
PXR	***Cyp3a3***	1.66 ± 0.65	3.01 ± 1.34** (1.8)	6.18 ± 2.66** (3.7)	8.72 ± 2.69** (5.3)	28.4 ± 7.22** (17.1)	83.7 ± 27.0** (50.4)
PPARα	***Cyp4a1***	0.78 ± 0.30	0.65 ± 0.25	0.69 ± 0.20	0.77 ± 0.16	0.63 ± 0.18	0.553 ± 0.08
Associated receptors	**Rat genes**	**28-Day recovery**					
AhR	***Cyp1a1***	0.72 ± 0.23	-	-	-	-	1.32 ± 1.11 (1.8)
CAR	***Cyp2b1***	0.30 ± 0.24	-	-	-	-	0.51 ± 0.36 (1.7)
PXR	***Cyp3a3***	2.67 ± 1.60	-	-	-	-	6.86 ± 3.75** (2.6)
PPARα	***Cyp4a1***	0.68 ± 0.28	-	-	-	-	0.76 ± 0.14 (1.1)

[a] Data were obtained from page 8 of the DER for MRID 49005910 (3 and 7 days of treatment); and pages 9 and 10 of the DER for MRID 49005902 (28 day treatment with a recovery group). Phenobarbital results are not included.

[b] This dose resulted in liver tumors in female rats (MRID 47372501).

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

( ) mean fold change relative to the controls.

Enzyme Activity

Table 5 presents the data showing rats exposed to fluopyram demonstrated a temporal and dose-related induction of CAR/PXR-related enzyme activities that corresponded with the previously presented gene expression data. PROD and BROD were significantly elevated at doses ≥ 600 ppm in the female rat as early as 7 days. PROD and BROD were also elevated at 150 ppm after 28 days, but the changes were not statistically significant. The slight increase in liver 7-ethoxyresorufin-O-deethylase (EROD) activity corresponds to the slight induction of *Cyp1a1* gene expression. Compared to levels immediately after 28-days dosing, enzyme activities were 67 to 96% less in animals allowed to recover for 28 days. All of these findings are consistent with results as presented in Table 6 from earlier studies (MRIDs 47372516 and 47372520).

Table 5. Mean Content or Enzyme Activity in Female Rats Following 7 Days or 28 Days of Treatment with Fluopyram.

Dose (ppm)	0 n = 5	30 n = 5	75 n = 5	150 n = 5	600 n = 5	1500 [c] n = 5
7 Day dosing [a]						
EROD	47.59 ± 8.52	50.09 ± 6.67	44.52 ± 6.27	48.58 ± 5.30	53.20 ± 5.63	77.47 ± 14.97* (1.6)
BROD	8.88 ± 0.26	9.65 ± 0.79	10.43 ± 0.84	12.81 ± 3.48	21.47 ± 12.24** (2.4)	52.30 ± 32.21** (5.9)
PROD	2.69 ± 0.22	3.60 ± 0.36	3.93 ± 0.86	3.78 ± 1.78	5.81 ± 2.64* (2.2)	12.27 ± 5.99** (4.6)
28 Day dosing [b]						
EROD	33.64 ± 3.92	37.97 ± 3.73	36.23 ± 7.43	45.20 ± 2.95** (1.3)	44.00 ± 3.91* (1.3)	66.07 ± 6.13** (2.0)
BROD	1.61 ± 0.78	2.00 ± 0.42	2.32 ± 0.63	4.65 ± 1.11 (2.9)	14.69 ± 11.18** (9.1)	62.93 ± 42.55** (39.1)
PROD	4.07 ± 0.34	3.75 ± 0.70	5.18 ± 0.63	6.22 ± 0.47 (1.5)	7.61 ± 2.24* (1.9)	19.36 ± 10.00** (4.8)
28 Day recovery [b]						
EROD	36.48 ± 3.23	-	-	-	-	44.02 ± 5.82* (1.2) [↓67%]
BROD	1.63 ± 0.35	-	-	-	-	2.39 ± 0.55* (1.5) [↓96%]
PROD	2.31 ± 0.61	-	-	-	-	3.56 ± 0.34** (1.5) [↓82%]

[a] Data were obtained from pages 7-9 of the DER for MRID 49005910.

[b] Data were obtained from page 9 of the DER for MRID 49005902, and phenobarbital results are not included.

[c] This dose resulted in liver tumors in female rats (MRID 47372501).

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

( ) fold change relative to the controls. [ ] % changes in recovery group relative to 28 day dosing values

Table 6. Enzyme Activity Shown as Fold Change Compared to Control for Male and Female Wistar Rats Exposed to Fluopyram for Either 28 Days or 7 Days (Females Only). [a]

	28 Days n=5						7 days n=10
	Males			Females			Females
Dose (ppm)	**50**	**400**	**3200**	**50**	**400**	**3200**	**3000**
EROD	-1.3	1.1	1.3	1.0	1.3	1.7	2.2*
PROD	-1.4	***4.5***	***10.4***	1.2	***4.6***	***16.3***	4.3*
BROD	1.1	***2.3***	***19.3***	1.5	***9.4***	***31.0***	11.7*

[a] Data were obtained from MRID 47372516 (BCS report SA 03332; 28 days) and MRID 47372520 (BCS report SA 07323; 7 days; females only).
* Significantly different from control, $p<0.05$
Bold Italics represent data considered as biologically significant (no statistical analyses conducted).

Associated effects supporting Key Event #1 (increased liver weights and liver hypertrophy)

Changes in liver weights and liver hypertrophy are associated effects that inform the degree of CAR/PXR-mediated hepatic events. Female rats exposed to fluopyram for 3, 7, or 28 days showed significantly increased liver weight from 150 ppm (Table 7). Following 90 days of treatment, liver weight continued to be increased at dose levels of 200 ppm and above; however following chronic treatment (12 or 24 months), statistically significant increases were only recorded in the high dose (1500 ppm) groups. Hypertrophy was observed in all studies starting from 400 ppm (MRID 47372516; Table 8). These effects (weight increase and hypertrophy) were completely reversible in rats treated at 1500 ppm fluopyram for 28 days followed by a 28-day recovery period on a control diet. In addition, hepatocellular hypertrophy was found to be reversible for rats exposed to 3200 ppm fluopyram for 90 days followed by a 28 day recovery period. These data show both a temporal- and dose-response.

Table 7. Increased Liver Weights in Female Rats (% Increase Over Controls)

Dose ↓ / Temporal →	Dose (ppm)	3 Days MRID 49005910	7 Days MRID 49005910	28 Days MRIDs 49005902 & 47372516	90 Days MRID 47372441	28 Days Recovery MRID 49005902	12 Months MRID 47372501	24 Months MRID 47372501
	30	0%	-5%	2%			1%	-3%
	50			0%	0%			
	75	2%	-3%	5%				
	150	4%	-2%	7%			4%	6%
	200				11%*			
	400			15%[a]				
	600	5%	3%	13%*				
	1000				27%*			
	1500[b]	17%*	18%*	33%*			54%*	56%*
	3200			73%*	74%*			

[a] It was considered treatment-related but was not statistically significantly different from controls.
[b] This dose resulted in liver tumors in female rats (MRID 47372501).
Blank cell = No data.
* Significantly different from control, $p<0.05$

Table 8. Temporal Dose-Response for Hepatocellular Hypertrophy in Female Rats (% Incidence). [a]

Dose ↓ / Temporal →	Dose (ppm)	3 Days MRID 49005910	7 Days MRID 49005910	28 Days MRIDs 49005902 & 47372516	90 Days MRID 47372441	28 Days Recovery MRID 49005902	12 Months MRID 47372501	24 Months MRID 47372501
	30	0	0	0			0	0
	50			0	0			
	75	0	0	0				
	150	0	0	0			0	0
	200				0			
	400			20*				
	600	0	7	40*				
	1000				70*			
	1500	40*	93*	93*		0	100*	81*
	3200			100*	100*	0		

[a] Data were obtained from page 24 of MRID 49005912.
Blank cell = No data.
* Significantly different from control, $p<0.05$

ii. Key Event #2: Hepatocellular proliferation

The available data show that typically, CAR/PXR inducers such as phenobarbital increase hepatocellular proliferation within 2 to 3 days of treatment then slowly return to "normal" levels of proliferation after about 4 - 6 weeks of exposure (slightly longer for mice; Kolaja *et al.*, 1996a; Yamada *et al.*, 2009).

In a preliminary study, female rats were exposed to 3000 ppm fluopyram for 7 days, and a significant increase in liver proliferation (increased 4x) was recorded (measured by BrdU incorporation; MRID 47372520). The current studies (MRIDs 49005902 and MRID 49005910) evaluated hepatocellular proliferation in female rats by Ki-67 immunohistochemical staining of labeled nuclei as a measure of hepatocellular proliferation (Table 9).

The greater degree of proliferation at 3 days versus 7 days shows the typical early wave of proliferation that peaks at around 3 days for fluopyram as for other known CAR/PXR inducers. Proliferation is slower between 3 and 7 days because the liver has already reached an enlarged (hepatomegaly) state and is adapting to adequately meet metabolic/detoxification demand. The threshold for significant induction of hepatocellular proliferation at 3, 7, or 28 days was ≥ 150 ppm. The significant proliferation recorded at ≥ 150 ppm after 28 days of exposure shows fluopyram affects proliferation in a sustained manner which is supported by the recovery data.

The recovery data for animals placed on the control diet for 28 days following a 28-day exposure at 1500 ppm demonstrate that the proliferative effects induced by fluopyram were not completely reversible. In the 1500 ppm group, total hepatocellular proliferation was 51% higher than control at the end of the recovery period and was approximately 50% lower than the amount of proliferation observed after 28 days of continuous treatment.

Table 9. Mean Cell Proliferation Indices in Female Rats Following 3, 7, or 28 Days of Treatment with Fluopyram.

Dose (ppm)	0 n=5	30 n=5	75 n=5	150 n=5	600 n =5	1500 n = 5
Day 3						
Centrilobular	14.6 ± 7.4	11.8 ± 8.0	13.4 ± 5.9	25.1 ± 11.1*	57.3 ± 20.1**	99.5 ± 62.3**
Perilobular	11.1 ± 5.1	10.7 ± 5.8	15.4 ± 8.2	22.6 ± 13.4**	36.6 ± 15.7**	67.4 ± 30.0**
Total	12.8 ± 5.8	11.2 ± 6.4 (-13%)	14.4 ± 5.8 (12%)	23.8 ± 10.6** (86%)	47.0 ± 15.8** (267%)	83.4 ± 38.3** (551%)
Day 7						
Centrilobular	8.3 ± 5.3	11.2 ± 7.6	12.0 ± 6.1	20.4 ± 13.0**	27.8 ± 12.1**	32.2 ± 19.6**
Perilobular	10.5 ± 6.1	15.3 ± 11.2	10.3 ± 5.1	18.4 ± 8.1**	27.0 ± 13.0**	34.9 ± 17.2**
Total	9.4 ± 5.1	13.2 ± 8.6 (40%)	11.1 ± 5.1 (18%)	19.4 ± 9.6** (106%)	27.4 ± 9.8** (192%)	33.5 ± 15.0** (257%)
Day 28						
Centrilobular	4.93 ± 3.11	4.23 ± 2.42	7.23 ± 3.55*	10.16 ± 3.86**	10.14 ± 5.27**	15.54 ± 7.33*
Periportal	8.37 ± 4.75	7.62 ± 3.66	8.51 ± 3.86	12.51 ± 3.97	12.10 ± 8.36	22.80 ± 10.49

Table 9. Mean Cell Proliferation Indices in Female Rats Following 3, 7, or 28 Days of Treatment with Fluopyram.						
Dose (ppm)	**0 n=5**	**30 n=5**	**75 n=5**	**150 n=5**	**600 n =5**	**1500 n = 5**
Total	6.65 ± 3.19	5.93 ± 2.82 (-11%)	7.87 ± 2.65 (18%)	11.33 ± 3.30** (70%)	11.12 ± 6.50** (67%)	19.17 ± 7.20** (189%)
Recovery phase						
Centrilobular	4.59 ± 2.44	-	-	-	-	8.30 ± 3.75**
Periportal	8.25 ± 4.60	-	-	-	-	11.12 ± 6.87
Total	6.42 ± 3.29	-	-	-	-	9.71± 4.77* (51%)

[a] Data were obtained from page 7 of the DER for MRID 49005910 and page 8 of the DER for MRID 49005902.
* Significantly different from control, p<0.05
** Significantly different from control, p<0.01
( ) % change from the controls

Table 10 summarizes the data presented in Table 10, and also demonstrates a dose-response relationship. A temporal relationship appeared to be present at 600 and 1500 ppm demonstrating the idea that peak hepatocellular proliferation occurred within 2 to 3 days of treatment then gradually decreased. After the 28 day recovery period the mean hepatocellular proliferation was still 51% higher than the controls.

Table 10. Temporal Dose-Response for Total Hepatocellular Proliferation in Female Rats Presented as % Change from the Control.					
	Temporal →				
Dose ↓	**Dose (ppm)**	**3 Days**	**7 Days**	**28 Days**	**28 day Recovery**
	30	-13	40	-11	
	75	12	18	18	
	150	86*	106*	70*	
	600	286*	192*	67*	
	1500	551*	257*	189*	51*

[a] Data were obtained from page 21 of the MOA submission (MRID 49005912)
* Significantly different from control, p<0.05

iii. Key Event #3: Altered Hepatic Foci

The chronic administration of CAR/PXR inducers, such as phenobarbital, leads to the development of altered hepatic foci (IARC, 2001; Jones *et al.*, 2009; Thorpe and Walker, 1973; Whysner *et al.*, 1996). These hepatic focal lesions are characterized by altered cytoplasmic tinctorial properties that can be classified as either basophilic, eosinophilic, clear cell, or mixed type (reviewed in Goodman *et al.*, 1994). Altered hepatic foci are classified as proliferative, preneoplastic lesions that can result from sustained hepatocellular proliferation. The liver lesions

produced by PXR-CAR inducer, phenobarbital, were predominantly eosinophilic in nature. This observation is also seen with fluopyram.

Hepatic foci are believed to be precursors of liver tumors because most rodent liver carcinogens increase their size and/or number prior to the appearance of tumors (Popp and Goldsworthy, 1989). Phenobarbital administration results in a dose-dependent increase in cell proliferation within foci that is associated with progression from altered hepatic foci to hepatocellular adenomas (Klaunig, 1993). Table 11 shows the incidence of altered hepatic foci observed at the conclusion of the carcinogenicity studies (24 months) for fluopyram were significantly greater than controls both in the female and the male rat (81 and 48 % occurrence at the top dose, respectively). In the rat chronic study (12 months), the incidence of altered hepatic foci was higher in the male compared to the female (50 and 30%, respectively). However, after 21 months on study, the high dose of 750 ppm demonstrated adverse clinical signs and mortality in the male rat required lowering of this dose to 375 ppm to ensure sufficient numbers of surviving males at the scheduled sacrifice to allow appropriate statistical analyses. This resulted in equivalent altered hepatic foci in males at 24 months (50 versus 48%), whereas an increase from 30 to 81% was recorded for the female rat (maintained at 1500 ppm fluopyram) at 12 and 24 months, respectively. If the male rats could have continued the carcinogenicity study at 750 ppm, it is highly probable that they would have developed liver tumors as was seen in the female rat.

Table 11. Incidence of Hepatocellular Alteration: Eosinophilic Foci (Focal/Multifocal). [a]

Sex		**Males**				**Females**			
12 Months	**Dose (ppm)**	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
	Incidence of altered hepatic foci	2/10 (20%)	1/10 (10%)	2/10 (20%)	5/10 (50%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	3/10 (30%)
24 Months	**Dose (ppm)**	**0**	**30**	**150**	**375**	**0**	**30**	**150**	**1500**
	Incidence of altered hepatic foci	16/60 (27%)	24/60 (40%)	31/60* (52%)	28/58** (48%)	29/60 (48%)	26/60 (43%)	30/60 (50%)	48/58** (81%)

[a] Data were obtained from MRIDs 47372501 and 49005912.

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

Summary of Liver MOA

The relevant molecular and pathological endpoints for fluopyram-induced liver effects are summarized below and are consistent with the established key events of nuclear receptor-mediated (CAR/PXR) rodent hepatocarcinogenesis (Cohen, 2010).

Liver Key Event #1

Key event #1 for the fluopyram-induced liver tumor MOA is defined as activation of CAR/PXR, which is measured by surrogate liver-specific induction of the CAR-specific Cyp2b and PXR-specific Cyp3a cytochrome gene, protein, and correlative PROD, BROD/BQ enzymatic activity.

Supportive, associative events to Key Event #1 include increased liver weight and microscopic hepatocellular hypertrophy. This key event occurs rapidly (significant changes are observed following 3 days of treatment) and has been well characterized in terms of dose-response. In addition, gene expression, enzyme activity, liver weight, and hepatocellular hypertrophy were reversible in rats after 28 days without exposure to fluopyram. In addition, PXR-CAR KO mice showed almost no induction of Phase I enzyme activity and no evidence of liver enlargement or hepatocellular hypertrophy after 28 days exposure to fluopyram at a dose level that was biologically comparable in terms of mg/kg/d (125 mg/g) to the rat the dose that caused tumors level (89 mg/kg).

Liver Key Event #2

Key event #2 is an increase in global hepatocellular proliferation that was seen in a dose-related manner in rats starting at 150 ppm, which was reversible following a 28-day recovery period (73% of the levels seen at 28 days). This finding is also consistent with the known MOA for CAR/PXR-mediated rodent liver tumorigenesis.

Liver Key Event #3

Key event #3 is an increase in altered hepatic foci, which are considered to be proliferative pre-neoplastic lesions (Jones *et al.*, 2009). Persistent hepatocellular proliferation leads to the induction of these lesions within the liver that can develop over time into adenomas and carcinomas (Cohen, 2010). A significant increase in altered hepatic foci was recorded at the top dose of fluopyram at the end of both the chronic (12 months) and carcinogenicity phase (24 months) of the cancer bioassay.

Generally, all of the key events for the MOA described for CAR/PXR-induced liver tumor were identified in fluopyram-exposed rats in a temporal- and dose-responsive manner with the final event of liver tumors observed in female rats exposed to 1500 ppm fluopyram.

b. Thyroid Tumor MOA and Key Events

A consequence of increased liver metabolism in rodents exposed to CAR/PXR inducers is increased elimination of thyroid hormones, which are produced by the thyroid and monitored by the hypothalamus-pituitary-thyroid axis. The hypothalamus releases thyrotropin-releasing hormone (TRH), which stimulates the anterior pituitary gland to secrete thyroid-stimulating hormone (TSH). TSH induces the thyroid gland to produce and release thyroxine (T4) and triiodothyronine (T3). The expression of TSH and TRH is controlled through a negative-feedback process that is very sensitive to circulating T4 and T3 levels. When the liver is exposed to high doses of a CAR/PXR inducer, it responds by increasing the size and number of cells as well as the activity of those enzymes capable of detoxifying the compound. Some of these detoxification enzymes, in particular the Phase 2 enzymes uridine diphosphate glucuronyltransferases (UDPGTs) and sulfotransferases, conjugate the CAR/PXR inducer with respectively a glucoronide or a sulfate moiety. This conjugation allows an easier elimination of the compound *via* urinary and biliary clearance (Klaassen and Hood, 2001). A consequence of this increased UDPGT and sulfotransferase activity is not only the increased elimination of

xenobiotics but also the increased conjugation and subsequent elimination of T4, resulting in decreased serum thyroid hormone levels. As part of a feedback mechanism, the pituitary increases its secretion of TSH in order to stimulate the thyroid gland to increase production of thyroid hormones to restore homeostasis. A sustained increase in thyroid hormone production is often achieved through hypertrophy and proliferation of thyroid follicular cells. After chronic exposure to high enough dose of a CAR/PXR inducer, hyperplasia and eventually tumors may develop in the thyroid gland due to sustained over-stimulation by TSH (Hiasa *et al.*, 1982; McClain *et al.*, 1988; Dellarco *et al.*, 2006;).

Based on these data, the following key events for thyroid tumor MOA were proposed by the Registrant, and the new mechanistic studies were conducted to support the proposed key events.

Key Events for CAR/PXR-Mediated Thyroid MOA (Dellarco et al., 2006)

(1) CAR/PXR receptor activation as demonstrated by induction of Phase I liver metabolic enzymes. Reversible upon discontinuance of treatment. Confirmation for CAR/PXR receptor activation provided by the results of PXR/CAR KO mice.
(2) Increased serum T4 clearance due to induction of Phase II liver metabolic enzymes. Reversible upon discontinuance of treatment
(3) Increased TSH level as measured by *TSH ß*. Reversible upon discontinuance of treatment
(4) Increased thyroid cell proliferation; reversible upon discontinuance of treatment.
(5) Thyroid Tumor formation

i. <u>Key Event #1: Liver CAR/PXR activation with induction of Phase I metabolic enzymes</u>

The first key event is activation of CAR/PXR nuclear receptors. However, no data are available to show direct interaction between CAR/PXR receptor and fluopyram. As described earlier, evidence for this first key event can be demonstrated by increased expression of specific hepatic cytochrome P450 genes and activation of their associated enzyme products.

Table 12 clearly shows increases in the mouse liver enzymes (PROD and BQ) specific for CAR and PXR at 30 ppm and higher. The increases also show a dose-related response. Activity levels for these enzymes returned to essentially control levels when treatment at the dose level of 750 ppm was followed by a 28-day recovery period on a control diet. Confirmation of CAR/PXR activation was established by comparing WT and PXR-CAR-KO mice; PROD was increased 1.4-fold in KO mice compared to 69.8-fold in WT mice, whereas BQ activity was reduced (0.68-fold) in KO mice compared to an induction of 5.5-fold in WT mice (Tables 13 and 14). These results clearly demonstrate the link between CAR/PXR activation and subsequent induction of Phase I enzymes.

In addition, the results from the study with WT and PXR-CAR-KO mice exposed to 750 ppm fluopyram for 28 days demonstrated a significant increase in liver weight in the WT, whereas

only a slight but significant increase was seen in KO mice (Table 16). In addition, enlarged liver and hepatocellular hypertrophy was observed in WT but not in the KO mice.

Table 12. Phase 1 (PROD and BQ) Enzyme Activities for Male Mice Treated with Fluopyram in the Diet for 28 Days (Including a Recovery Group).

Dose (ppm)	0 n = 5	30 n = 5	75 n = 4	150 n = 5	600 n = 5	750 [b] n = 5
28 Day dosing						
PROD (pmol formed/ min/mg protein)	4.65 ± 1.12	67.22 ± 6.95** (14)	156.89 ± 46.37** (34)	170.99 ± 29.09*** (37)	201.51 ± 45.02** (43)	219.06 ± 31.31** (47)
BQ (nmol formed/ min/mg protein)	7.59 ± 0.67	10.68 ± 1.92* (1.4)	16.71 ± 2.61** (2.2)	21.98 ± 1.76** (2.9)	39.20 ± 7.49** (5.2)	47.24 ± 3.85** (6.2)
Recovery phase						
PROD (pmol formed/ min/mg protein	4.63 ± 0.82	-	-	-	-	5.27 ± 0.75 (1.1)
BQ (nmol 7- formed/ min/mg protein)	6.93 ± 1.01	-	-	-	-	6.68 ± 1.27 (NC)

[a] Data were obtained from page 8 of the DER for MRIDs 49005911 and 49005903.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

( ) Value in the parenthesis indicates folds change relative to the control. NC = no change relative to the control.

Table 13. Phase 1 (PROD and BQ) Enzyme Activities for Wild Type (WT) and PXR/CAR KO Male Mice Following 28-Days of Treatment with Fluopyram.

Dose (ppm)	0 n = 15	750 n = 15	1500 n = 15	0 n = 15	750 [b] n = 15	1500 n = 15
	C57BL/6J (WT)			**PXR /CAR KO**		
PROD (pmols formed/min/ mg protein	2.01 ± 0.20	140.21 ± 15.11** (69.8)	302.14 ± 84.76** (150)	2.27 ± 0.30	3.20 ± 0.81** (1.4)	3.24 ± 1.16** (1.4)
BQ (nmols formed/min/ mg protein)	2.77 ± 0.34	15.20 ± 1.89** (5.5)	21.94 ± 1.83** (7.9)	3.51 ± 0.39	2.40 ± 0.40** (0.68)	2.09 ± 0.35** (0.60)

[a] Data were obtained from page 7 of the DER for MRID 49005906.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

( ) Value in parenthesis indicated fold change relative to the control.

Table 14. Mean Liver to Body Weight Ratio, Enlarged Liver, and Hepatocellular Hypertrophy in Wild Type and PXR-CAR Knockout Mice Exposed to Fluopyram for 28 Days. [a]				
Mouse	**Wild Type (n= 15)**		**PXR-CAR Knock out (n= 15)**	
Dose (ppm)	**0**	**750**	**0**	**750**
Mean liver to bw ratio (% increase)	0	39*	0	7*
Enlarged liver (% increase)	0	*47**	0	0
Hepatocellular hypertrophy (% occurrence)	0	*100**	0	0

[a] Data were obtained from MRID 49005906.
* Significantly different from control, $p<0.05$
Italics considered biologically significant.

ii. Key Event #2: Phase II liver enzyme induction leading to increased serum T_4 clearance and consequently decreased circulating T_4

Following CAR/PXR activation, liver metabolic enzymes that conjugate and eliminate thyroid hormones are induced. T_3 and T_4 are inactivated in the liver mainly by UDPGT-mediated conversion to glucuronide derivatives, which are eliminated via urine and bile (Rutgers *et al.*, 1989). This results in reduced serum T_4 concentrations in both mice (Hood *et al.*, 2003) and rats (Hood *et al.*, 1999; Liu *et al.* 1995). The activation of these Phase II enzymes is directly responsible for the cascade of events producing lower serum T_4, increased TSH, elevated thyroid follicular cell proliferation, and eventually thyroid tumors (Barter and Klaassen, 1992; Hurley *et al.*,1998; Dellarco *et al.*, 2006). The activities of two UDPGT isoenzymes, UDPGT-T4 and UDPGT-BIL, were evaluated in the mouse mechanistic studies.

Table 15 shows an increase in the activity of both of UDPGT-T_4 and UDPGT-BIL starting from 150 ppm. Both UDPGT-T_4 and UDPGT-BIL levels returned to the control level following a 28 day recovery period. The registrant claimed that the absence of a statistically significant response for UDPGT-T_4 at 750 ppm was due to assay variability, because in an independent study (MRID 49005906) of an identical design, a 1.8-fold increase (statistically significant) in UDPGT-T_4 activity was observed in WT mice (Table 15). In contrast, the activity measured in PXR-CAR-KO mice was essentially comparable to untreated control group (Table 16).

Table 15. Mean Enzymatic UDPGT Activities Following 28 Days of Fluopyram Treatment in Male Mice and Following a Recovery Phase. [a]						
Dose (ppm)	**0 n = 30-15 [c]**	**30 n = 15**	**75 n = 15**	**150 n = 15**	**600 n = 15**	**750 [b] n = 30-15 [c]**
28 Days						
UDPGT-T_4	0.77 ± 0.14	0.77 ± 0.19 (NC)	0.85 ± 0.20 1.1	1.17 ± 0.36 1.5	1.41 ± 0.25** 1.8	1.03 ± 0.27 (1.3) (1.8)[a]
UDPGT-BIL	1.98 ± 0.58	2.22 ± .038 (1.1)	2.39 ± 0.33 (1.2)	2.62 ± 0.20 (1.3)	2.76 ± 0.32* (1.4)	2.95 ± 0.42** (1.5)
Recovery phase						
UDPGT-T_4	0.82 ± 0.12	-	-	-	-	0.77 ± 0.33 (0.93)

UDPGT-BIL	1.80 ± 0.31	-	-	-	-	1.91 ± 0.24 (1.1)

[a] Data were obtained from pages 7 of the DER for MRIDs 49005911 and 49005903. In an independent study of an identical design, a 1.8-fold increase (statistically significant) in UDPGT-T4 activity was observed.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

[c] 30-15 represents a total of 30 test animals in this dose group with 15 of the 30 test animals being placed in the recovery group.

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

Table 16. Enzyme Activity Examining Phase II (UDPGT-T_4 and UDPGT-BIL) Enzyme Activity in Male C57BL/6J (Wild Type) and PXR-CAR Knockout (KO) Mice Exposed to Fluopyram in the Diet for 28 Days.

Dose (ppm)	**0 n = 15**	**600 n = 14**	**750 [b] n = 15**	**0 n = 15**	**600 n = 15**	**750 [b] n = 15**
	C57BL/6J			**PXR KO/CAR KO**		
UDPGT-T_4 (nmol T_4 glucuronide formed/min/ mg protein	0.58 ± 0.17	1.06 ± 0.17** (1.8)	1.09 ± 0.25** (1.9)	0.57 ± 0.19	0.66 ± 0.17 (1.2)	0.43 ± 0.15* (0.75)
UDPGT-BIL (nmol bilirubin-glucuronide formed/min/ mg protein	0.73 ± 0.11	1.30 ± 0.22** (1.8)	1.43 ± 0.42** (1.8)	0.70 ± 0.24	0.66 ± 0.24 (0.86)	0.61 ± 0.28 (0.87)

[a] Data obtained from page 31-32 of MRID 49005906.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

( ) Value in the parenthesis = fold change relative to the control.

Following the increase in Phase II enzymes, plasma T_4 concentration is expected to decrease. Several mouse studies were conducted to determine the effects of fluopyram treatment on circulating thyroid hormone levels. When mice were exposed to fluopyram at concentrations of 100 and 300 ppm (below the dose that caused tumors, 750 ppm) for 3 days, there was a noticeable and statistically significant drop in plasma T_4 (Table 17). A significant decrease in T_4 was also observed at Day 3, when male mice were exposed to 2000 ppm fluopyram (MRID 47372519; Table 18).

Table 17. Mean Plasma Thyroxin (T4) Values in Male Mice Following 3 Days of Fluopyram Treatment. [a]

Dose (mg/kg bw/d)	**0 n = 15**	**100 n = 15**	**300 n = 15**
T_4 (nmol/L)	34.2 ± 8.7	25.4 ± 6.1** (↓26%)	22.6 ± 4.6** (↓34%)

[a] Data were obtained from Table 3, page 4 of the DER (MRID 49005909)

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

Table 18. Mean Plasma Thyroid Hormone Levels in Male Mice. [a]				
	3 days		14 Days	
Dose (ppm)	**0**	**2000**	**0**	**2000**
T_3	1.62 ± 0.15	1.64 ± 0.25 (+1%)	1.45 ± 0.18	1.52 ± 0.38 (+ 5%)
T_4	43.7 ± 8.1	30.7± 6.0** (-30%)	38.1 ± 9.1	27.7 ± 8.7** (-27%)

[a] Data were obtained from page 25 of MRID 47372519.

iii. Key Event #3: Increased TSH

The first two key events leading to CAR/PXR-induced thyroid alterations occur in the liver; however, as previously discussed that reduced circulating levels of T_4 can activate the hypothalamic-pituitary-thyroid axis feedback mechanism in an attempt to maintain thyroid hormone homeostasis. Serum T_4 and T_3 concentrations are monitored by the hypothalamus and the anterior pituitary gland. A decrease in serum thyroid hormone concentrations stimulates the hypothalamus to secrete TRH, which then stimulates the release of TSH from the anterior pituitary. Increased pituitary secretion of TSH stimulates the function and the growth of the thyroid gland, resulting in an increased production of thyroid hormones, T_4 and T_3, to restore thyroid hormone homeostasis.

Due to difficulties in detecting clear changes in TSH plasma levels in early studies (MRIDs 49005909 and 49005901), it was decided to measure pituitary "thyroid- stimulating hormone, beta" (TSH β*)* transcript levels in the later studies. *TSH β* is a gene that provides instructions for making a beta protein subunit that is specific of TSH. When thyroid hormone levels are low, the hypothalamus produces TRH, which stimulates the pituitary gland to produce more TSH β (needed to produce more TSH). Thus, an increase in TSH β mRNA can serve as a biomarker for increased TSH protein levels. EPA agreed that TSH β could be considered as a biomarker for TSH. In a 28-day study (MRID 49005911) examining TSH β in mice, a significant induction of this transcript was observed in the pituitary gland at 600 and 750 ppm fluopyram (Table 19). During the recovery phase, the level of this transcript returned to the control level.

Table 19. Mean Relative TSH Transcript Levels Following 28 Days of Treatment and a Recovery Phase. [a]						
Dose (ppm)	**0 n = 30-15**	**30 n = 15**	**75 n = 15**	**150 n = 15**	**600 n = 15**	**750 [b] n = 30-15**
28 Days dosing						
TSH β	1.16 ± 0.33	1.22 ± 0.35 (+6%)	1.30 ± 0.34 (+13%)	1.30 ± 0.45 (+12%)	**1.66 ± 0.46* (+43%)**	**1.78 ± 0.72** (+54%)**
Recovery phase						
TSH β	1.05 ± 0.09	-	-	-	-	1.17 ± 0.18* (+12%)

[a] Data were obtained from pages 30-32 of MRID 49005911.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, p<0.05
** Significantly different from control, p<0.01

Support that CAR/PXR activation is required to produce changes in TSH transcript levels is provided when examining the gene expression of *TSH ß* in PXR-CAR-KO mice treated with fluopyram, as no change was seen in transcript levels in the KO mice (Table 20). The results with the KO mice provide evidence that, without the activation of CAR/PXR, UDPGT enzyme activity is not elevated (Tables 15 and 16), and there is no perturbation of the pituitary-thyroid axis.

Table 20. Mean Relative Quantity of Transcript Following 28 Days of Treatment in Wild Type and Knock Out (KO) Mice. [a]

	C57BL/6J			PXR/CAR KO		
Dose (ppm)	**0 n = 15**	**750** [b] **n = 15**	**1500 n = 15**	**0 n = 15**	**750** [b] **n = 15**	**1500 n = 15**
TSH ß	1.23 ± 0.289	1.92 ± 0.306** (+56%)	2.05 ± 0.586** (+67%)	1.25 ± 0.264	1.14 ± 0.177	1.04 ± 0.165* (-17%)

[a] Data were obtained from pages 32-33 of MRID 49005906.
[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).
* Significantly different from control, p<0.05
** Significantly different from control, p<0.01

iv. Key Event #4: Increased Thyroid Cell Proliferation

The data presented previously showed that exposure to fluopyram results in decreases in thyroid hormone, T_4. The demand for increased thyroid hormones by TSH is met by increased thyroid follicular cell proliferation. This process of thyroid follicular cell proliferation, if sustained for a prolonged period of time, can result in thyroid gland tumors (McClain, 1992). The hypothesis of sustained proliferation leading to tumorigenesis is similar to that described for the liver.

In a study in mice which received fluopyram for 28 days, a dose-related increase in thyroid follicular cell proliferation was seen starting from 150 ppm (Table 21). This effect was reversible after cessation of treatment (1500 ppm) followed by 28 days on a control diet. Furthermore, when WT and CAR-PXR-KO mice treated at 750 or 1500 ppm, a significant increase was observed in the thyroid cell proliferation index for WT mice, whereas the treated KO mouse group was similar to the KO control (Table 22). The absence of increased cellular proliferation in KO mice demonstrates that the activation of the CAR/PXR receptor is obligatory for the induction of the thyroid follicular cell alterations in mice exposed to fluopyram.

Table 21. Thyroid Cell Proliferation Index in Male Mice Following 28 Days of Treatment and a Recovery Phase. [a]							
Dose (ppm)	**0**	**30**	**75**	**150**	**600**	**750** [b]	**1500**
Dosing for 28 days (n=15)							
Rate/1000 cells	21.55 ± 4.75	17.81 ± 7.37	19.51 ± 5.64	26.09 ± 8.62 (+21%)	**30.11 ± 8.53** (+40%)**	**34.78 ± 7.61** (+61%)**	**50.21 ± 10.24** (+133%)**
Recovery phase (n=14)							
Rate/1000 cells	17.57 ± 5.18	-	-	-	-	-	11.56 ± 4.78 (-34%)

[a] Data were obtained from pages 26-27 of MRID 490059505.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, p<0.05.

** Significantly different from control, p<0.01

Table 22. Thyroid Gland Proliferation Index Following 28 Days of Treatment. [a]						
Dose (ppm)	**0 n = 15**	**750 n = 15**	**1500 n = 15**	**0 n = 15**	**750 n = 15**	**1500 n = 15**
	C57BL/6J (WT)			PXR KO/CAR KO		
Proliferation index	14.3 ± 3.96	26.1 ± 7.16** (+86%)	36.6 ± 10.27** (+156%)	10.05 ± 3.88	9.91 ± 4.06 (-1%)	8.27 ± 3.38 (-18%)

[a] Data were obtained from page 30 of MRID 49005906.

* Significantly different from control, p<0.05

** Significantly different from control, p<0.01

v. Key Event #5: Increased Thyroid Cell Hyperplasia

In male mice exposed to 2000 ppm fluopyram for 3 or 14 days, no evidence of gross- or microscopic changes to the thyroid gland was observed (MRID 47372519). Furthermore, no adverse thyroid findings were noted in the 28- and 90-day mouse studies at up to 5000 and 1000 ppm, respectively. Increased incidence of thyroid follicular cell hyperplasia was observed at ≥150 ppm in males at 12 and 18 months and at 750 ppm in females at 18 months (Table 23). The data on the fluopyram-induced thyroid follicular cell hyperplasia demonstrate that pre-neoplastic cellular changes in the thyroid may require exposure of 12 months or longer.

Table 23. Thyroid Follicular Cell Hyperplasia in the Male Mice Exposed to Fluopyram for 12 or 18 Months. [a]

		Males			
	Dose (ppm)	**0**	**30**	**150**	**750**
12 months	**Thyroid follicular cell hyperplasia**	0/9 (0%)	0/10 (0%)	2/9 *(22%)*	2/10 *(20%)*
18 months	**Thyroid follicular cell hyperplasia**	4/50 (8%)	6/50 (12%)	21/50** (42%)	32/50** (64%)
		Females			
18 months	**Thyroid follicular cell hyperplasia (%)**	17/48 (35%)	8/50 (16%)	19/50 (38%)	33/50* (66%)

[a] Data were obtained from pages 10 and 12 of MRID 47372450.

[b] This dose resulted in tumors in males in the mouse carcinogenicity study (MRID 47372450).

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

Italics represents biologically significant in the absence of statistical significance

Mouse Thyroid Associative Events

Increased liver weight and hypertrophy played a key role in the formation of CAR/PXR-induced thyroid tumors. In the standard 28 and 90 day mouse studies, significant findings of increased liver weight and hepatocellular hypertrophy were seen at ≥ 150 ppm (Table 24, liver weight; Table 25, liver hypertrophy).

In a study with WT and PXR-CAR-KO mice exposed to 750 and 1500 ppm fluopyram for 28 days, a significant increase in liver weight was observed in the WT, whereas only a slight but significant increase was seen in KO mice (Table 26). In addition, hepatocellular hypertrophy and enlarged liver were observed in WT but not in the KO mice. Overall, these data support the idea that the liver induction in male mice is likely to be associated with the thyroid tumor formation, reinforcing that fluopyram induces thyroid tumors indirectly via the liver CAR/PXR activation as the first key event.

Table 24. Temporal Dose-Response for Increased Relative Liver Weights in Male C57BL/6J Mice Given as % Increase Over Controls.

Dose ↓ / Temporal →	ppm	3 days MRID 47372519	14 days MRID 47372519	28 days MRID 49005911 & 47372517	90 days MRID 47372442	12 months MRID 47372450	24 months MRID 47372450
	30			5%		0%	8%
	75			7%			
	150			11%*		15%*	15%*
	600			27%*			
	750 (Recovery)			33%* (4%)*		25%*	27%*
	1000						
	2000	59%*	59%*				

Blank cell = No data.
* Significantly different from control, $p<0.05$
MRIDs 49005911 and 47372517 have common dose levels

Table 25. Temporal Dose-Response for Hepatocellular Hypertrophy in Male C57BL/6J Mice Given as % Occurrence.

Temporal →

Dose ↓	ppm	3 days MRID 47372519	14 days MRID 47372519	28 days MRID 47372517	90 days MRID 47372442	12 months MRID 47372450	24 months MRID 47372450
	30				0	0	0
	150			**100%**	**100%**	**63%**	**78%**
	750			**100%**		**100%**	**100%**
	1000				**100%**		
	2000	**100%**	**100%**				

Blank cell = No data. **Bold** indicates treatment-related and biologically significant in absence of statistical analyses.

Table 26. Mean Liver Weights, Enlarged Liver, and Hepatocellular Hypertrophy in Male Wild Type and PXR-CAR Knockout (KO) Mice Exposed to Fluopyram for 28 Days. [a]						
	C57BL/6J (n=15)			PXR KO/CAR KO (n=15)		
Dose (ppm)	**0**	**750**	**1500**	**0**	**750**	**1500**
Liver Weight	1.28 ± 0.069	1.80 ± 0.159** (↑41%)	2.12 ± 0.191** (↑66%)	1.29 ± 0.080	1.40 ± 0.080** (↑9%)	1.43 ± 0.077** (↑9%)
Enlarged Liver	0	7/15 (47%)	14/15 (93%)	0	0	0
Hepatocellular Hypertrophy	0	15/15 (100%)	15/15 (100%)	0	0	0

[a] Data were obtained from pages 27-28 of MRID 49005906.

* Significantly different from control, $p<0.05$

** Significantly different from control, $p<0.01$

Summary of Thyroid MOA

The MOA for fluopyram-induced rodent thyroid effects is similar to that for known CAR/PXR inducers. The use of CAR/PXR knockout mice has demonstrated that fluopyram is a CAR/PXR-inducer producing typical liver effects and thyroid alterations. The relevant molecular and pathological endpoints for fluopyram-induced liver and thyroid effects appear to be consistent with the established key events of CAR/PXR-mediated rodent thyroid tumorigenesis (Dellarco *et al.*, 2006; Hurley *et al.*, 1998). The key events are summarized below.

Thyroid Key Event #1

Key event #1 for fluopyram-induced thyroid tumor MOA is defined as activation of CAR/PXR, as observed by increases in the Phase I enzymes PROD (specific for CAR) and BQ (specific for PXR). On cessation of treatment, enzyme activities returned to control levels. As expected in PXR-CAR-KO mice, only marginal changes in PROD and BQ were observed.

Thyroid Key Event #2

Key event #2 is defined as UDPGT induction leading to increased T_4 clearance and decreased plasma T_4. In mice, the induction of the UDPGT-associated enzymes was not seen until ≥ 150 ppm. On cessation of fluopyram exposure, enzyme levels returned to normal. Additionally, PXR-CAR-KO mice showed no induction of UDPGT-associated Phase II enzymes after exposure to a dose that caused tumors for 28 days. Indications of increased T_4 clearance were observed in mice pre-exposed to fluopyram at 2000 ppm for up to 4 days prior to injection with radiolabeled T_4. Further evidence for effects on T_4 levels was provided by several studies in which decreases in plasma T_4 were observed.

Thyroid Key Event #3

Key event #3 is an increased level of TSH. A significant induction of *TSH ß* in the pituitary gland of mice was observed at ≥ 600 ppm following fluopyram exposure for 28 days. Following cessation of treatment for 28 days, the levels of this transcript returned to normal. When *TSH ß*

was examined in PXR-CAR-KO mice exposed to a carcinogenic concentration of fluopyram for 28 days, no change in transcript levels was seen, consistent with the lack of change in levels of UDPGT-associated enzymes. This finding shows that activation of CAR and PXR in the liver is obligatory to induce thyroid alterations following exposure to fluopyram.

Thyroid Key Event #4

Key event #4 is an increase in thyroid follicular cell proliferation. A dose response increase in follicular cell proliferation was observed in mice exposed to fluopyram for 28 days starting from 150 ppm. However, PXR-CAR-KO mice exposed to the dose that caused tumors for 28 days showed no increase in follicular cell proliferation, demonstrating that activation of these receptors is required for altered thyroid effects.

Thyroid Key Event #5

Key event #5 is an increase incidence of thyroid follicular cell hyperplasia seen after chronic administration of fluopyram to mice. The effect was observed at ≥ 150 ppm. Overall the key events for CAR/PXR-induced thyroid tumors were identified in fluopyram-exposed mice in a temporal and dose-responsive manner, with the final event of thyroid adenomas observed in male mice exposed to 750 ppm fluopyram. The absence of the key events in PXR-CAR-KO mice exposed to fluopyram provides strong support for CAR/PXR being the molecular initiating event for these tumors.

Overall the key events for CAR/PXR-induced thyroid tumors were identified in fluopyram-exposed mice in a temporal and dose-responsive manner, with the final event of thyroid adenomas observed in male mice exposed to 750 ppm fluopyram. The available mechanistic data provide strong support for CAR/PXR being the molecular initiating event for the thyroid tumors, and this is confirmed by the absence of the key events in PXR-CAR-KO mice exposed to fluopyram.

c. Strength, Consistency, and Specificity of Association of Tumor Response with Key Events

In the available mechanistic data, the key events observed following exposure to fluopyram in both the mouse and rat occurred in a biologically relevant temporal sequence, were dose-dependent, and took place at dose levels that were at or below the doses that produced tumors.

Liver tumor MOA— Key events for fluopyram-induced rat liver tumor MOA were defined as: 1) activation of CAR/PXR, 2) increased hepatocellular proliferation, and 3) increased altered hepatic foci. All of these key events were identified, key events 1 and 2 were shown to be reversible, and all had a dose- and temporal-response.

Thyroid tumor MOA—The key events for fluopyram-induced mouse thyroid tumors were defined as: 1) activation of CAR/PXR, 2) induction of UDPGT (with an indication for increased T4 clearance and decreased plasma T4 levels), 3) increased TSH (*TSH β*), 4) increased thyroid follicular cell proliferation, and 5) increased thyroid follicular cell hyperplasia. All of these key

events were identified, characterized in terms of dose and temporal response, and were shown to be reversible. As summarized in Table 27, key events 1 through 4 were absent in CAR-PXR-KO mice exposed to 750 ppm fluopyram the dose that caused tumors. This fact supports that the liver CAR /PXR activation is the initial molecular event causing the thyroid tumors in male mice.

Table 27. Comparison of Fluopyram-Induced in Wild Type and PXR-CAR Knockout Male Mice Treated with 750 ppm Fluopyram in the Diet for 28 Days. [a]

Key Events	Parameter	Wild-type	PXR-CAR-KO
1	Hepatic Phase 1 enzyme activity	↑PROD/BQ	No change
2	Hepatic Phanse II enzyme activity	↑UDPGT-T_4/ UDPGT-Bil	No change
3	TSH ß	↑	No change
4	Thyroid follicular cell proliferation	↑	No change

[a] Data were obtained from MRID 49005906.

d. Biological Plausibility and Coherence

Based on the available data, the proposed MOA for liver tumors in female rats and thyroid tumors in male mice after exposure to fluopyram is considered biologically plausible and coherent. The proposed key events are mostly consistent with the published literature information on the non-genotoxic mitogenic liver carcinogen. The common initial key event is the activation of rodent CAR and PXR, which produces a cascade of alterations in gene transcription that leads to increased hepatic metabolizing enzyme activities. In rats, the cascade of alterations led to hepatocellular proliferation, a critical event in the development of liver tumors. In male mice, the data showed that alterations in gene transcription led to increase in hepatic Phase II enzyme activities, which resulted in another series of events leading to thyroid follicular cell proliferation. The early key events of hepatic enzyme induction and cellular (liver and thyroid) proliferation, as well as the associative events of increased liver weight and hepatocellular hypertrophy, were largely reversible on cessation of treatment. Finally, the specificity of the MOA was demonstrated for fluopyram by using a genetically engineered PXR-CAR-KO mouse model. Fluopyram treated PXR-CAR-KO mice did not demonstrate PXR-CAR mediated hepatic or thyroid effects observed in wildtype mice. These data are consistent with the known MOA for phenobarbital and other PXR-CAR activators. Summaries of the analyses are presented in Tables 28 and 29.

Table 28. Analysis of Fluopyram Rat Liver Tumor MOA.

Key Event #1: CAR/PXR receptor activation; w/ associated CYP enzyme induction; w/ associated liver hypertrophy Key Event #2: Hepatocellular proliferation Key Event #3: Altered hepatic foci Key events 1 and 2 were shown to be reversible after cessation of fluopyram treatment.	
Strength of association	+
Consistency of association	+
Specificity of association	+
Dose response concordance	+

Temporal relationship	+
Coherence & plausibility	+ & +

+: Attribute present

Table 29. Analysis of Fluopyram Mouse Thyroid Tumor MOA.	
Key Event #1: CAR/PXR receptor activation; w/ associated CYP enzyme induction Key Event #2:Phase II liver enzyme induction and decrease T_4 Key Event #3. Increased TSH Key Event #4. Thyroid follicular cell proliferation Key Event #5. Increased thyroid follicular cell proliferation Key events 1, 2, 3, & 4 are reversible and they are not demonstrated in fluopyram treated PXR-CAR KO-mice.	
Strength of	+
Consistency of association	+
Specificity of association	+
Dose response concordance	+
Temporal relationship	+
Coherence & plausibility	+ & +

+: Attribute present

e. Alternative Modes of Action for Liver Tumors

i. Mutagenic (DNA Reactivity

There is no evidence from a comprehensive battery of genotoxicity assays of any mutagenic, clastogenic, aneugenic or DNA reactive activity of fluopyram. Based on the lack of genotoxicity in the available studies, a mutagenic mode of action is not supported.

ii. Cytotoxicity/Regenerative Cell Proliferation

No indication of hepatic damage or cytotoxicity was see in the rat, the fluopyram-induced tumor do not appear to be acting through a cytoxic MOA.

iii. Activation of the Peroxisome Proliferator-Activated Receptor Alpha (PPARα)

An increase in *Cyp4a1* level is considered an indicator of Pparα activation. Mechanistic studies examined liver *Cyp4a1* transcript level in fluopyram-treated male rats at different durations and dose levels. The results demonstrate mostly decreases in the levels of *Cyp4a1* transcript, as shown in Table 30. Therefore, the data do not support that this receptor plays a role in the key events leading to fluopyram-induced liver tumors.

Table 30. Levels of *Cyp4a1* Transcript (% of Control) in Female Rats Treated with Fluopyram at Various Dose Levels and Durations.					
Dose (ppm)	**30**	**75**	**150**	**600**	**1500**
3 Days	-6%	+3%	-11%	-2%	-22%
7 Days	-3%	-15%	-12%	-12%	-37%
28 Days	-17%	-12%	-1%	-19%	-29%

The % of the control values were calculated from the data presented in Table 4.

iv. Activation of the Aryl Hydrocarbon Receptor (AhR)

Activators of AhR include a variety of polycyclic aromatic hydrocarbons, including the chlorinated dioxins and related halogenated aromatic hydrocarbons. Fluopyram is not a polycyclic aromatic hydrocarbon, as it does not have fused aromatic rings and contains elements other than hydrogen and carbon (nitrogen, oxygen, fluorine, and chlorine). Furthermore, it is not a chlorinated dioxin; however, it can be classified as a halogenated aromatic hydrocarbon molecule, because of the presence of fluorine and chlorine. *Cyp1a1* is used as a sensitive, although non-specific, indicator of AhR binding and activation; whereas, EROD is used to examine the activity of this enzyme (Hu *et al.*, 2007). Evaluation of the *Cyp1a1* gene expression levels and EROD liver enzymatic activity from fluopyram-exposed animals is summarized in Table 31.

Table 31. *Cyp1a1* Gene Expression and EROD Enzyme Activity (Expressed as Fold Change, Compared to Control) in Female Wistar Rats Exposed to Fluopyram for up to 28 Days with a 28 Day Recovery High Dose Group. [a]					
Dose (ppm)	**30**	**75**	**150**	**600**	**1500**
	Cyp1a1				
3 days	-1.2	1.1	1.7	7.3**	62.7**
7 days	1.4	1.8	4.6**	63.6**	222.9**
28 days	1.8	2.3	8.1**	100.9**	354.7**
Recovery					1.8
	EROD				
7 days	1.1	0.9	1.0	1.1	1.6*
28 days	1.1	1.1	1.3**	1.3*	2.0**
Recovery					1.2

[a] Data were obtained from Tables 4 and 5 of this document.
* Significantly different from control, $p<0.05$
** Significantly different from control, $p<0.01$

Although there was a large increase in *Cyp1a1* transcript at the top dose of 1500 ppm (355-fold) at 28 days, this did not translate into a similar magnitude of increased enzyme activity, as EROD was only elevated 2-fold. These results show a discordant response with a large induction of *Cyp1a1* gene transcript and a very minimal increase in EROD enzyme activity in the rat following exposure to fluopyram. In addition, the registrant reported that in other studies examining EROD levels in rats exposed to the known AhR agonist β-naphthoflavone, EROD levels were increased 9.1-fold, while EROD levels in rats treated with fluopyram remain consistently lower than those seen for β-naphthoflavone – despite the highest dose of fluopyram

being more than 3 times higher (Table 32). In evaluating these data, it is unlikely that fluopyram is a significant agonist or activator of AhR. The minimal/mild effects seen on AhR markers here may be due to cross-talk subsequent to the significant induction of CAR and PXR.

Table 32. EROD Enzyme Activity in Male and Female Wistar Rats Exposed to Dietary Fluopyram for 28 Days. Additionally, EROD Levels for the Positive Control, β-Naphthoflavone, Exposed via Oral Gavage at 75 mg/kg/day for 28 Days. [a]

	Males				Females			
Dose (ppm)	50	400	3200		50	400	3200	
Dose (mg/kg/day)	4	31	254	ß-NF: 75	4.6	36.1	263	ß-NF: 75
EROD (fold change relative to controls)	-1.3	1.1	1.3	7.2	1.0	1.3	1.7	9.1

[a] Data were obtained from page 56 of MRID 49005912.
ß-NF = ß-naphthoflavone

v. Activation of the Estrogen Receptor (ER)

Estrogens have a specific receptor-mediated MOA that results in cell proliferation in tissues, including the liver; however, fluopyram is not likely to have an estrogenic MOA based on its structural dissimilarity to estrogens. Most importantly, in the standard rat and mouse studies and the two-generation rat study, no evidence of interference in the estrogen system was observed, *e.g.,* decreased fertility in males, alterations in male and female reproductive organ weights, estrous cyclicity, or precocious vaginal opening. Additionally, fluopyram was not a developmental or reproductive toxicant. The available developmental toxicity studies in rats and rabbits and the multi-generation reproduction study in rats demonstrate no evidence of increased qualitative or quantitative susceptibility in developing or young animals following exposure during pre- or post-natal periods. From these data, it is concluded that fluopyram does not act as an agonist or activate the ER, and thus this receptor would not play a role in the development of fluopyram-induced tumors.

vi. Statins

The rodent profile for a statin-induced liver tumor MOA consists of an increase in liver HMG-CoA-reductase, *Cyp2b* and *Cyp4a* transcript levels, and hepatocellular proliferation and no change in serum cholesterol (Kocarek and Reddy, 1996; Cohen, 2010). No measure of HMG-CoA-reductase was conducted in the course of the fluopyram mechanistic work. Fluopyram was shown to increase serum cholesterol concentrations and mildly suppress *Cyp4a1* gene expression, both of which are opposite findings to those seen following exposure to known statins. These data demonstrate that fluopyram is not acting as a statin in rodents, and this MOA is unlikely to be responsible for fluopyram-induced tumors.

vii. Apoptosis

Standard regulatory studies do not typically provide quantitative data on the incidence of apoptosis in the liver or other tissues, and short-term mechanistic studies are not lengthy enough to induce altered hepatic foci for apoptosis evaluation. In addition, hepatocellular toxicity can

occur not only secondary to apoptosis but also from an increase in necrosis. Either or both of these findings can result in regenerative proliferation and, if sustained, in the development of liver tumors. With respect to fluopyram, no characterization of increased apoptosis was undertaken, because this was seen as only an associative event that does not inform the MOA of CAR/PXR activation (Cohen, 2010).

Finally a summary of possible alternative MOA for liver tumor is presented in Table 33.

Table 33. Summary of Possible Alternative MOAs for Fluopyram-Induced Liver Tumor Formation.

	DNA Reactivity (mutagenic)	**AhR or PPARα activation**	**Cytotoxicity**	**Increased Apoptosis**	**Estrogen Statins**
Strength of Association	All genotoxicity assays negative	No increase in relevant gene transcripts	No changes in Relevant clinical chemistry parameters &no hepatic focal necrosis	No histopathogical evidence. Difficult to determine microscopically	No histopathological evidence
Consistency of Association	–	–	–	–	–
Specificity of Association	–	–	–	–	–
Dose Response Concordance	No tumors in lower dose levels in rats	–	–	–	–
Temporal Relationship	Late onset tumors	–	–	–	–
Coherence & Plausibility	No Coherence Not Plausible	No Coherence Not Plausible	No Coherence Not Plausible	No Coherence Not Plausible	No Coherence Not Plausible

- Indicates attribute absent.

f. Alternative Modes of Action for Thyroid Tumors

The following alternative MOAs may be considered for thyroid tumor formation:

i. DNA reactivity.

Based on the available genotoxicity data, fluopyram was test negative in all genotoxicity assays. Therefore, alternative MOAs are not likely.

ii. Inhibition of the active transport of inorganic iodide into the follicular cell (iodide pump).

iii. Inhibition of thyroid peroxidase that converts inorganic iodide into organic iodide. And couples iodinated tyrosyl moieties into thyroid hormone.

iv. Inhibition of thyroid hormone release into the blood.

Alternative MOAs ii, iii, and iv are the direct thyroid gland effects and are not supported by the available data on fluopyram. The evidence of an increased T_4 clearance does not favor a direct effect of fluopyram on thyroid hormone biosynthesis and release to explain the decreased plasma T_4 levels induced by fluopyram. Moreover, mechanistic studies using hog thyroid microsomes showed fluopyram did not affect thyroid peroxidase (MRID 47372518). The findings in the KO mice as discussed below also confirm that fluopyram does not produce direct thyroid effects as postulated by these three alternative MOAs.

v. Damage to thyroid follicular cells.

Alternative MOA v, damage to thyroid follicular cells, is not supported by the available data, as histopathology data on the thyroid gland of mice and rats do not show overt cytotoxicity.

vi. Inhibition of the conversion of T4 to T3 by 5'-monodeiodinase at various sites in the body.

vii. Enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through the action of UDPGT.

Alternative mechanism vi, inhibition of the conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body, is unlikely because serum levels of T_3 were not changed in either mice or rats exposed to fluopyram. This indicates that MOA vii (enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UDPGT enzymes) is the most likely mechanism. Fluopyram increased the activity of UDPGTs, which catabolize T_4.

The results of PXR-CAR-KO mouse study demonstrate that fluopyram is a CAR-PXR activator and is not a direct thyroid toxicant. In this study, no induction of UGT-T4, no induction of *TSH* β transcripts, and no increased thyroid follicular cell proliferation was observed in KO mice following fluopyram treatment, whereas all these effects were observed in wild type mice. These data demonstrate that the interaction of fluopyram with hepatic CAR-PXR nuclear receptors is obligatory to induce hepatic Phase II and pituitary *TSH* β transcript and ultimately the pre-neoplastic thyroid effects. Table 34 summarizes the anticipated effects induced by PXR-CAR activator and direct thyroid toxicant. From all the possible MOAs that lead to thyroid hyperplasia and tumors, the available mechanistic data support the MOA involving hepatic PXR-CAR-nuclear receptor activation as the initial event in a cascade of events leading to thyroid tumors.

Table 34. Anticipated Effects Induced by Direct Thyroid Toxicant and PXR-CAR Activator in Wild Type and PXR-CAR-KO Mice. [a]				
	PXR-CAR activator (indirect)		**Direct thyroid toxicant**	
	Wild Type	PXR-CAR-KO	Wild Type	PXR-CAR-KO
UDPGT-T_4	↑	No change	No change	No change
T_4 clearance	↑	No change	No change	No change
Plasma T_4	↓	No change	↓	↓
TSH β transcripts	↑	No change	↑	↑
Thyroid follicular cell proliferation	↑	No change	↑	↑

[a] Data were obtained from page 65 of MRID 49005912.

g. Data Limitations, Uncertainties, and Inconsistencies

Lack of reliable TSH in the plasma

The Registrant stated that during the course of the mechanistic program it was technically challenging to obtain reliable measurements for plasma levels of T_4 and TSH. Although a dose related decrease in plasma T_4 levels due to fluopyram treatment was demonstrated in two oral gavage studies using high dose levels (100 and 300 mg/kg/day i.e. ~750 and ~2000 ppm, respectively [MRID 49005901, MRID 49005909]), a clear dose-response relationship could not be established when treatment was via the diet at doses ≤750 ppm. Several factors could have contributed to this observation, such as the fact that the hormone measurements were taken following dietary administration. With respect to plasma TSH measurements, it was difficult to identify alterations in TSH concentrations during the entire mechanistic program. This may be due to the fact that all thyroid-related effects induced by fluopyram were weak. Consequently, the Registrant used *TSH β* transcripts as a surrogate marker of plasma TSH and considers the increase in this biomarker as evidence for an increase in TSH.

h. Human Relevance

The CARC considers both liver and thyroid tumors produced by fluopyram to be potentially relevant to humans.

V. CONCLUSION AND CLASSIFICATION OF CARCINOGENIC POTENTIAL OF FLUOPYRAM

In 2009, fluopyram was classified as **"Likely to be Carcinogenic to Humans"** based on tumors in two species and two sexes: a treatment-related increase in thyroid follicular cell adenomas in high dose male mice and liver tumors in high dose female rats, with incidences exceeding that of the laboratory's historical controls. There is no mutagenic concern for fluopyram. The data supporting the previously proposed MOA were insufficient. Subsequently, the registrant conducted a series of mechanistic studies to support the currently postulated MOA for liver and thyroid tumor formation.

The additional data submitted were considered adequate to establish the mode of action for the etiology of these tumors. Key events leading to the progression towards liver tumors included sequentially the activation of the CAR/PXR receptors resulting in induction of hepatic cytochrome P450 activity, hepatocellular proliferation, altered hepatic foci, and liver tumors, altered hepatic foci, and liver tumors. These key events were established based on dose-response and temporal concordance at appropriate doses. Key events leading to the progression towards thyroid tumors included sequentially the activation of the CAR/PXR receptors resulting in induction of hepatic cytochrome P450 activity, induction of phase II hepatic enzymes resulting in increased serum T4 clearance, increased TSH, increased thyroid cell proliferation, increased thyroid cell hyperplasia, and thyroid tumors. These key events were established based on dose-response and temporal concordance at appropriate doses. Alternate modes of actions were also considered, but were rejected based on the observed toxicity and biological function.

The CARC considers the hypothesized mode of action (CAR/PXR receptor mediated, mitogenic) for liver and thyroid tumors is adequately supported by the currently available data. These data have clearly identified the sequence of key events, and have demonstrated dose-response concordance, and temporal relationship to tumor types. The CARC classifies fluopyram as "**Not Likely to be Carcinogenic to Humans" at doses that do not induce cellular proliferation in the liver or thyroid glands."** This classification is based on convincing evidence that a non-genotoxic mode of action for liver tumors in rats and thyroid tumors in mice has been established and that the carcinogenic effects have been demonstrated as a result of a mode of action dependent on activation of the CAR/PXR receptors.

VI. QUANTIFICATION OF CARCINOGENIC POTENTIAL

The CARC has determined that quantification of risk is not required. There is sufficient data to ascertain the mode of action of fluopyram. The chronic Reference Dose (RfD) is derived using the NOAEL of 1.2 mg/kg/day as the "point of departure" which is below the dose of 11 mg/kg/day that caused cell proliferation in the liver (i.e., a key event in tumor formation) and the subsequent liver tumors at a higher dose (89 mg/kg/day). Additionally, there is no concern for mutagenicity.

Attachment A

List of repeat dose and liver/thyroid specific mechanistic MOA Studies

MRID 47372516 Kennel, P. (2004); AE C656948 - Exploratory 28-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience , Sophia Antipolis, France; Report No.: SA 03332; Document No.: M-085510-01.

MRID 47372517 Kennel, P. (2004); AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 04013; Document No.: M-088486-01.

MRID 47372441 Kennel, P. (2005); AE C656948 – 90-day toxicity study in the rat by dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 04048; Document No.: M-250946-01.

MRID 47372442 Kennel, P. (2005); AE C656948 – 90-day toxicity study in the mouse by Dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 04052; Document No.: M-251136-01.

MRID 47372450 Wason, S. (2007); Carcinogenicity study of AE C656948 in the C57BL/6J Mice by dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 05094; Document No.: M-295688-01.

MRID 47372501 Kennel, P. (2008); Chronic toxicity and carcinogenicity study of AE C656948 in the Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 04312; Document No.: M-298339-01.

MRID 47372520 Blanck, M. (2008); Fluopyram (AE C656948) - 7-day mechanistic study in the female Wistar rat by dietary administration; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 07323; Document No.: M-299274-01.

MRID 47372523 Blanck, M. (2008); Phenobarbital - 7-day mechanistic study in the female Wistar Rat by gavage; Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France; Report No.: SA 07325; Document No.: M-299491-01.

MRID 47372518 Freyberger, A. (2008); AE C656948 (Fluopyram) - *In vitro* studies on the Potential interactions with thyroid peroxidase-catalyzed reactions; Bayer HealthCare AG, Wuppertal, Germany; Report No.: AT04481; Document No.: M-299276-01.

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